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# Mitochondrial DNA diversity and *Wolbachia* infection in the flea beetle *Aphthona nigriscutis* (Coleoptera: Chrysomelidae): An introduced biocontrol agent for leafy spurge

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**Abstract**

*Aphthona nigriscutis* is one of several species of *Aphthona* flea beetles that have been introduced into North America in an effort to control the weed, leafy spurge (*Euphorbia esula*). It has been a very effective biological control agent at some locations but not at others. Overall genetic diversity is one parameter that could have an effect on *Aphthona* establishment at specific locations. We have examined the genetic diversity of mitochondrial DNA in populations of *A. nigriscutis* from several North American collection sites. The results indicate that the insects are divided into two mtDNA clades. About 78% of the individuals comprise a clade (A) that has little or no mtDNA diversity. The remaining insects in the other clade (B) display extensive diversity with 15 haplotypes observed. The two subpopulations coexist at most locations. The bacterial endosymbiont *Wolbachia* has been discovered in some individuals. About 86% of the individuals from mtDNA clade A tested positive for *Wolbachia*. Portions of the *Wolbachia ftsZ* and *wspA* genes were sequenced and the sequences have been shown to fall within the *Wolbachia* Supergroup A. None of the insects from clade B appear to be infected. The association of *Wolbachia* with one, but not both, mtDNA clades of *A. nigriscutis* may play a role in limiting genetic diversity within beetle populations.
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## 1. Introduction

Several species of Chrysomelid flea beetles from the genus *Aphthona* have been introduced into North America since the late 1980s in an effort to control the invasive weed, leafy spurge (*Euphorbia esula* L.). Both the plants and the insects are natives of Eurasia. In a broad sense, biological control of leafy spurge has been quite effective, with an 80–90% reduction of spurge at sites where *Aphthona* flea beetles have become established (Anderson et al., 1999; Team Leafy Spurge). Four species account for almost all of the currently established populations, *Aphthona flava* Guillebeau, *Aphthona cyparissiae* Koch, *Aphthona lacertosa* Rosenheim, and *Aphthona nigriscutis* Foudras. The latter two are dominant at most established North American sites.

One downside that has become apparent as the beetle populations have been expanded in both natural dispersal and continued introductions is that they fail to establish at many spurge infested locations. This difficulty is more pronounced in *A. nigriscutis*. Species differences in habitat preferences and dispersal capabilities along with local variation

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in the spurge may explain some of the failures (Flaherty, 2001; Jonsen et al., 2001; Müller-Schärer et al., 2004; Mundal et al., 1999). The presence of a genetic bottleneck and a reduction in genetic diversity could also restrict the effectiveness of the beetles in certain environments or on certain strains of leafy spurge. Many insects imported as potential biological control agents are collected as small populations from one or few sites and may not reflect the full genetic diversity of the species. Consideration of the genetic diversity has the potential for making biological control agents more broadly effective (Hopper et al., 1993). Many of the *Aphthona* beetles redistributed in the central USA and Canadian prairies also come from a relatively small number of beetle recollection sites. A genetic bottleneck may have occurred at initial collection sites for importation and/or at collection sites for redistribution of *A. nigriscutis*, resulting in a narrowed genetic base that may limit beetle adaptability at new release sites.

Little is known about the inherent genetic variability of the *Aphthona* species, either as released populations in North America or as endemic species in Europe and Asia. Our overall goal is to assess the genetic variation of the *Aphthona* populations that have become established in the USA and Canada, beginning with an examination of mitochondrial DNA. There have been reports of *Wolbachia* infections in North American *A. nigriscutis* (Kazmer, 2001; Nowierski et al., 2001). *Wolbachia* infections can have a direct influence on reducing the diversity of mtDNA (Armbruster et al., 2003; Behura et al., 2001; Shoemaker et al., 2003). There are conflicting views regarding the possible extent to which *Wolbachia* may also affect the nuclear genetic diversity (Ballard et al., 2002; Telschow et al., 2002). Because *A. nigriscutis* is such an important biocontrol agent of leafy spurge, any factor that may affect its establishment and reproductive success is of interest. Therefore, we also tested the populations for the presence of *Wolbachia* and defined the *Wolbachia* that we found. This report details the mtDNA diversity from the species *A. nigriscutis*, the distribution of that diversity, and the impact of *Wolbachia* on that diversity.

## 2. Materials and methods

### 2.1. Insects

Adult *A. nigriscutis* were collected from established sites in North Dakota (ND), Minnesota (MN), and Alberta (AB), Canada. Insects from the USA were brought to Fargo live and frozen at −80 °C for future use. Insects from Canada were frozen in Canada and sent to Fargo packed in dry ice. The three ND collection sites were near Medora (MED) (lat: 46.9444, long: −103.5333) in southwestern ND, near Lisbon (LIS) (lat: 46.4166, long: −97.5000) in the southeastern part of the state, and in Ward County (WAR) (lat: 48.3083, long: −101.3333) near Minot in north central ND. The Minnesota collections were from the Minnesota River Valley in Eden Prairie and Shakopee near the Minneapolis-St. Paul metropolitan area. RIV is near Grass Lake (lat: 44.81807, long: −93.45514), FCL is by the Flying Cloud Airport (lat: 44.81895, long: −93.47341) (both north of the river), and BLU is at Blue Lake (lat: 44.79591, long: −93.43922) which is south of the river. The ALB4 site is in southern Alberta, Canada about 20 km southwest of Lethbridge, AB (lat: 49.584167, long: −112.871111). *A. nigriscutis* was incidental at two other sites in southern Alberta, ALB1 (lat: 49.244722, long: −113.260278) and ALB3 (lat: 49.181389, long: −113.308333).

The most frequent *Aphthona* species found at these sites was *A. lacertosa*, but *A. cyparissiae* and *A. flava* could sometimes be present in small numbers. Initial sorting separated the black beetles, *A. lacertosa*, from all of the others which are brown. Specimens, as numbered sample exemplars, were submitted to Dr. Fauske for species identification. Species determinations were based upon external morphology, internal morphology, and genitalic dissection. Previously identified mtDNA clades were than assigned to species based upon matching sequences in the morphologically determined specimens.

### 2.2. DNA preparation and PCR

Total beetle DNA was extracted from either whole insects or the head and thorax portion via the high salt procedure of Cheung et al. (1993). Alternatively, DNA was extracted from one of the hind legs using the DNeasy Tissue kit (Qiagen, Valencia, CA). When only a partial insect was used, the remainder of the insect was returned to the freezer at the Biosciences Research Laboratory in Fargo as a voucher specimen. The mitochondrial primers 16S2 (LR-N-12945 5′-GCGACCTCGATGTTGGATT) and C2R (C2-J-3684 5′-GGTCAATGTTCAGAAATTTGTGG) were used to amplify a portion of the mtDNA about 9 kb in length. Reaction components were from the Applied Biosciences XL kit and the long PCR conditions and mtDNA primers were as described previously (Roehrdanz, 1995; Roehrdanz and Degrugillier, 1998). The PCR products were digested with the restriction endonucleases *Xba*I, *Ase*I, *Alu*I, *Ssp*I, *Dra*I, *Dpn*II or *Hin*fI. Verification of the proper sized amplicons and the restriction fragment length polymorphism (RFLP) patterns were made using agarose gel electrophoresis and ethidium bromide staining in the company of molecular size standards. Individual RFLP patterns were given an alphabetical designation while the composite haplotypes were numbered in the order they were discovered. Some initial haplotype numbers in sequence were discarded when those individuals were determined to belong to one of the other existing haplotypes.

### 2.3. Wolbachia

The presence of *Wolbachia* was determined by conducting PCR with primers specific for *Wolbachia* genes. The primers ftsZ-1 (5′-GTTGTCGCAAATACCGATGC) and ftsZ-2 (5′-CTTAAGTAAGCTGGTATATC) amplify about 1050 bp of the *fts*Z cell cycle protein. *Fts*Z-F (5′-TACTGACTGTTGGAGTTGTAACTAAGCCGT) and *fts*Z-R (5′-TGCCAGTTGCAAGAACAGAAACTCTAACTC) amplify about 570 bp in the middle of the larger *fts*Z segment (Werren et al., 1995b). An approximately 600 bp piece of *wsp*A, a *Wolbachia* surface protein gene, is produced with the primers *wsp*A-81F (5′-TGG TCCAATAAGTGATGAAGAAACTAGCTA) and *wsp*A-691R (5′-AAAAATTAAACGCTACTCCAGCTTCTGCAC) (Zhou et al., 1998). Representatives of all three gene segments were cloned and sequenced. DNA sequencing was done by the Iowa State University DNA sequencing facility. GenBank Accession Nos. are AY136550 and AF539858–AF539861.

*2.4. Data analysis*

The genetic relationships of the *A. nigriscutis* RFLP haplotypes were determined using the RestSite software (Miller, 1991) which relies on Nei's measure of genetic distance. The software has the advantage of being able to analyze restriction fragment data generated by enzymes with different recognition site lengths in a single operation. Bootstrapping is employed within the program but specific bootstrap numbers are not part of the output. The genetic distance matrix from RestSite was used for tree construction both within RestSite and also with the Neighbor and Drawgram programs of PHYLIP (Felsenstein, 1993). *Wolbachia* sequences were compared to other sequences in the NCBI database to determine to which of the major *Wolbachia* Supergroups this *Wolbachia* belongs. The *Wolbachia* sequences were trimmed to comparable lengths and aligned with Vector NTI AlignX (InVitrogen).

## 3. Results

The PCR amplicon used for RFLP analysis comprises about 60% of the mitochondrial genome. The seven restriction enzymes yielded 35 restriction patterns. *Dra*I had the fewest patterns with three. *Dpn*II had the most with seven. Approximately, 90 restriction fragments were scored for each haplotype (Table 1) and 16 different haplotypes were identified (Table 2). Most of the haplotypes were rare. Only five of the haplotypes were recovered in four or more of the 214 insects tested. The rare haplotypes were not concentrated at any particular location. Excluding the ALB1 and ALB3 sites, all of the locations had more than one haplotype, with two sites having six haplotypes. The widespread occurrence of divergent haplotypes reflects that this species contained measurable genetic variability in the original European collections and that establishment in North America has not eliminated genetic diversity. The overall diversity far exceeds what could have reasonably occurred in the 10–15 years since the original releases. Haplotype N1 is the dominant haplotype. It was found at every site, including the single insects at ALB1 and ALB3. Fully 78% of the *A. nigriscutis* tested were haplotype N1 (Table 3). *A. nigriscutis* was common at all of the flea beetle established sites except the two Canadian sites, ALB1 and ABL3. At those two locations only 1/62 and 1/73, respectively, were *A. nigriscutis*, however 60–80% of the beetles were black (*A. lacertosa*) and the minority brown beetles were predominantly *A. cyparissiae*. By contrast site ALB4 had only brown beetles with 70% being *A. nigriscutis*. The mtDNA haplotypes from *A. nigriscutis* separate into two major clades, A and B, with about 2% divergence between the clades (Fig. 1). Haplotype N1 is the exclusive member of clade A. The remaining 15 haplotypes make up the other branch, clade B with <1% intra-clade divergence.

All but one of the insects whose mtDNA was evaluated were tested for the presence of *Wolbachia* using one of the *fts*Z primer sets. In addition, 170 of the insects were also tested with the *wsp*A primer set. All insects testing positive for *Wolbachia* using the *fts*Z primer set also tested positive with the *wsp*A primers. Approximately 2/3 of the insects tested positive for the presence of *Wolbachia*. *Wolbachia* infection was restricted to one of the mtDNA haplotypes; all of the *Wolbachia* infected individuals were haplotype N1 (Clade A). The overall infection rate for haplotype N1 is 86%. At sites where more than 10 individuals were tested, infection rates ranged from 60% at LIS to 94% at WAR. None of the 46 insects in Clade B tested positive for infection.

The nucleotide sequences of the *fts*Z and *wsp*A amplicons were determined from several individuals. Three *fts*Z sequences of 570 bp, obtained from one Ward County (2927) and two Medora (2594 and 2599) beetles, were identical. A 605 bp segment of the *wsp*A gene was sequenced from two Medora beetles (2595 and 2599) and one from Ward County (2927). Beetle 2595 had a substitution at position 371 that results in an amino acid change. A substitution at position 517 of beetle 2599 does not affect the amino acid sequence. Comparison of the *A. nigriscutis Wolbachia* with other sequences in GenBank places both gene segments firmly in the *Wolbachia* Supergroup A (data not shown). Neither the *fts*Z nor the *wsp*A sequences were an exact match to any other *Wolbachia* sequences currently in the database. The closest match to *fts*Z from another insect differs by five bases and comes from the European raspberry beetle (*Byturus tomentosus* DeGeer, Byturidae, GenBank AJ250964).

The sex was determined for the insects that only a hind leg was used for the DNA preparation. Some of the populations had remaining insects that were not used for DNA and a sample of these was also used for sex determination. All 18 FCL beetles examined were female, with 17 being Clade A and 1 Clade B. Among 38 insects from ALB4, 36 were female and only 2 were males. All were haplotype N1. *Wolbachia* was detected in 20 of the females and both of the males. Twenty-two beetles from RIV were all female. Among these, 17 were Clade A and all harbored *Wolbachia*. Five were Clade B and none had *Wolbachia*. From the BLU site 18 beetles were sexed resulting in 16 females and 2 males. 12/16 females are haplotype N1 (Clade A) and 10 of them have *Wolbachia*. The four other females and the two

Table 1
*Aphthona nigriscutis* restriction fragment patterns for 16S2-C2R amplicon

| | |
|---|---|
| *Xba*I | Size in base pairs |
| A. | >5000, 1100, 625, 450, 300 |
| B. | >5000, 625, 551, 550, 450, 300 |
| C. | <4500, 1600, 625, 551, 550, 450, 300 |
| D. | 4500, 1600, 1050, 550 |
| G. | >5000, 625, 550, 450 |
| H. | <4500, 1600, 625, 551, 550, 450 |
| *Ase*I | Size in base pairs |
| A. | 650, 575, 550, 490, 460, 400, 375, 355, 320, 290, 255, 240, 200, 175, 160, 130, 115 |
| B. | 650, 620, 575, 550, 475, 460, 400, 375, 355, 320, 290, 245, 200, 175, 160, 130, 115 |
| C. | 620, 575, 550, 475, 460, 400, 375, 355, 320, 290, 245, 200, 175, 160, 130, 115 |
| D. | 650, 620, 575, 551, 550, 475, 460, 400, 375, 290, 245, 235, 200, 175, 160, 130, 115 |
| E. | 750, 650, 575, 550, 490, 460, 400, 375, 355, 320, 290, 255, 240, 200, 175, 160, 130, 115 |
| *Alu*I | Size in base pairs |
| A. | 1400, 870, 830, 650, 525, 510, 485, 420, 340, 280, 270, 230, 160, 150, 120, 100 |
| B. | 1350, 870, 830, 650, 525, 510, 485, 420, 340, 280, 270, 230, 160, 150, 120, 100 |
| C. | 1350, 870, 830, 650, 525, 510, 485, 420, 340, 280, 270, 230, 185, 160, 150, 120, 100 |
| E. | 1350, 1100, 830, 650, 525, 510, 485, 420, 280, 270, 185, 160, 150, 140, 120, 100 |
| *Ssp*I | Size in base pairs |
| A. | 1600, 770, 510, 480, 420, 395, 360, 350, 300, 280, 250, 210, 200, 190, 110, 75 |
| B. | 1450, 770, 750, 510, 480, 420, 395, 360, 350, 300, 280, 265, 210, 200, 190, 110, 75 |
| C. | 1450, 770, 510, 480, 420, 395, 360, 350, 300, 280, 265, 250, 210, 200, 110 |
| D. | 1450, 770, 750, 510, 480, 420, 395, 360, 350, 300, 280, 265, 240, 210, 200, 190, 110, 75 |
| *Dra*I | Size in base pairs |
| A. | 1550, 950, 900, 840, 700, 425, 335, 300, 270, 240, 225, 190, 150, 125 |
| B. | 840, 730, 700, 650, 480, 440, 335, 300, 270, 240, 225, 190, 150, 125 |
| C. | 950, 900, 840, 730, 700, 650, 335, 310, 300, 285, 270, 240, 225, 190, 150, 125 |
| *Dpn*II | Size in base pairs |
| A. | 3300, 1000, 750, 650, 560, 525, 450, 410, 220, 200, 110, 100 |
| B. | 3300, 1250, 1000, 750, 560, 525, 500, 450, 220, 180 |
| C. | 4000, 2100, 1000, 560, 500, 450, 220, 180, 110 |
| D. | 4000, 2300, 1000, 560, 500, 450, 220 |
| E. | 3300, 1000, 750, 560, 525, 480, 450, 410, 220, 180 |
| F. | 4000, 1500, 1150, 1000, 560, 500, 220 |
| G. | 3800, 1150, 1000, 560, 525, 450, 420, 220, 180,110, 90 |
| *Hin*fI | Size in base pairs |
| A. | 2800, 2300, 1400, 525, 475, 380, 350, 200, 165, 140 |
| B. | 2600, 1600, 1400, 1150, 525, 450, 380, 260, 165, 140 |
| C. | 2600, 1600, 1400, 825, 650, 450, 380, 350, 165, 140 |
| D. | 2600, 1600, 1400, 750, 525, 450, 380, 350, 165, 140 |
| E. | 2600, 1450, 1400, 770, 525, 450, 380, 350, 260, 165, 140 |
| F. | 2600, 1600, 1400, 1150, 525, 450, 380, 350, 165, 140 |

males belong to Clade B and do not have *Wolbachia*. Overall 96 beetles comprised 92 females and 4 males.

## 4. Discussion

The mtDNA results point to a genetically divided group of *A. nigriscutis*, a majority group that is monotypic for mtDNA and a minority group with extensive mtDNA diversity. The genetic distance between the two groups is >2%. This compares to about 4.5–6.0% distance between the known species. The 2% level suggests a period of reproductive isolation that could be a manifestation of the *Wolbachia* infection or the result of different geographical origin locations. We have found no intermediates linking the two groups. There is evidence from mites that *Wolbachia* can trigger assortative mating of infected versus uninfected individuals (Vala et al., 2003). We do not have any information to indicate whether assortative mating is taking place within the subpopulations. Although the two groups appear to coexist at most locations, it should be pointed out that there are no historical data to establish whether one of these two subpopulations is gaining at the expense of the other. Given the >2% genetic separation of the two clades, we cannot exclude the possibility that *A. nigriscutis* comprises a species complex and the two clades represent cryptic species.

Overall the results from *A. nigriscutis* do not suggest a severe genetic bottleneck occurred during its introduction into North America. The level of genetic diversity is far greater than what would arise in the 10–15 years since introduction. Although there are no available data for the European populations, the logical inference is that the

Table 2
mtDNA haplotypes in *A. nigriscutis*

| Haplotype[b] | Restriction digestion patterns[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Xba*I | *Ase*I | *Alu*I | *Ssp*I | *Dra*I | *Dpn*II | *Hinf*I |
| N1 | A | A | A | A | A | A | A |
| N2 | D | B | C | B | B | B | B |
| N3 | C | C | C | B | C | C | C |
| N4 | B | C | B | B | C | D | D |
| N5 | C | B | C | B | B | B | B |
| N6 | D | B | B | B | B | B | B |
| N7 | B | C | C | B | C | C | C |
| N8 | C | C | B | B | C | C | C |
| N9 | C | D | B | B | B | B | B |
| N11 | C | B | C | B | B | F | B |
| N12 | H | B | C | B | B | B | F |
| N13 | C | B | C | B | C | C | C |
| N14 | C | B | E | B | B | B | B |
| N15 | G | B | C | D | B | G | B |
| N16 | C | B | B | B | B | B | B |
| N19 | C | B | E | B | C | F | E |

[a] Restriction patterns as described in Table 1.
[b] N# = *A. nigriscutis* haplotype number. See Section 2.

diversity existed in Europe. The records associated with the introductions concur to the extent that original releases did not derive from a few tens of insects. In 1989 a total of 1111 *A. nigriscutis* that had been collected from Baja, Hungary were released by APHIS at seven sites in the USA (mean = 158/site). Since some insects were used for identification, or placed on potted plants during quarantine, or died in transit a conservative estimate of 1400–1500 insects in the original collections seems reasonable. APHIS repeated the collection and importation in 1990 (probably with similar numbers although the records are currently not available). In 1989 and 1990, APHIS also released 200–1000 beetles per site at a number of locations. These insects came from Cardston, Alberta, Canada where they had been established in the middle 1980s from collections also made near Baja, Hungary (Hansen et al., 1997; R Hansen, personal communication). Over the entire time period, a substantial number of *A. nigriscutis* were imported to North America although they were obtained from a limited geographic range. Various state and university groups imported and released beetles at other locations during this same time period.

*Wolbachia* is an intracellular bacteria with widespread distribution among insects, crustaceans, and nematodes (Stouthamer et al., 1999; Werren, 1997). Depending on the detection methodology, *Wolbachia* has been found in 20–76% of insects (Jeyaprakash and Hoy, 2000; Jiggins et al., 2001; Werren et al., 1995a). *Wolbachia* is associated with reproductive disruptions including cytoplasmic incompatibility (CI), parthenogenesis induction, feminization of males, and killing of male embryos (Stouthamer et al., 1999; VanBorm et al., 2001). There is ample evidence of *Wolbachia* infections spreading and replacing uninfected populations. The discovery of *Wolbachia* in the monotypic mtDNA clade gives us some clues about how the separation of that clade might have arisen and been maintained. Two of the more easily recognized population consequences associated with *Wolbachia* infections are a loss of mtDNA diversity associated with infection and a possible distortion of the sex ratio in favor of females. *Wolbachia* infection is restricted to clade A which has no observed intra-clade mtDNA diversity. That clade accounts for about 2/3 of the beetles averaged over a wide area. The uninfected clade B has considerably more mtDNA variability, a total of 15 haplotypes. Since both *Wolbachia* and

Table 3
Number of *A. nigriscutis* haplotypes at each collection site and *Wolbachia* infection status of each haplotype

| Haplotype[a] | Collection site and date[b] | | | | | | | | | W+ | W− | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MED 6/28/01 | LIS 6/25/02 | WAR 8/8/02 | RIV 6/18/03 | FCL 6/18/03 | BLU 6/18/03 | ALB1 7/03 | ALB3 7/03 | ALB4 7/03 & 7/04 | | | |
| N1 | 2(2)[c] | 15(9) | 72(68) | 12(9) | 17(13) | 8(8) | 1(1) | 1(1) | 41(34) | 144 | 24 | 168 |
| N2 | | 2 | 6 | | 1 | 1 | | | | | 10 | 10 |
| N3 | | 1 | | 2 | | 6 | | | | | 9 | 9 |
| N4 | 2 | | | | | | | | | | 2 | 2 |
| N5 | | 2 | 6 | | | | | | | | 8 | 8 |
| N6 | | | 1 | | | | | | | | 1 | 1 |
| N7 | | | 1 | | | | | | | | 1 | 1 |
| N8 | 4 | | | | | | | | | | 4 | 4 |
| N9 | | | 1 | 1 | | | | | | | 2 | 2 |
| N11 | | | | | | | | | 2 | | 2 | 2 |
| N12 | | | | | | | | | 2 | | 2 | 2 |
| N13 | | | | | | 1 | | | | | 1 | 1 |
| N14 | | | | | | 1 | | | | | 1 | 1 |
| N15 | | | | | | 1 | | | | | 1 | 1 |
| N16 | 1 | | | | | | | | | | 1 | 1 |
| N19 | | | | | | | | | 1 | | | 1 |
| Total | 8 | 20 | 87 | 15 | 18 | 18 | 1 | 1 | 46 | 144 | 69 | 214 |
| %N1 | 12 | 75 | 83 | 80 | 94 | 44 | 100 | 100 | 89 | 67 | 33 | |

W+, infected; W−, uninfected.
[a] Haplotype numbers as in Table 2.
[b] Collection sites are described in Section 2.
[c] Number in parentheses is the number that tested positive for *Wolbachia*.

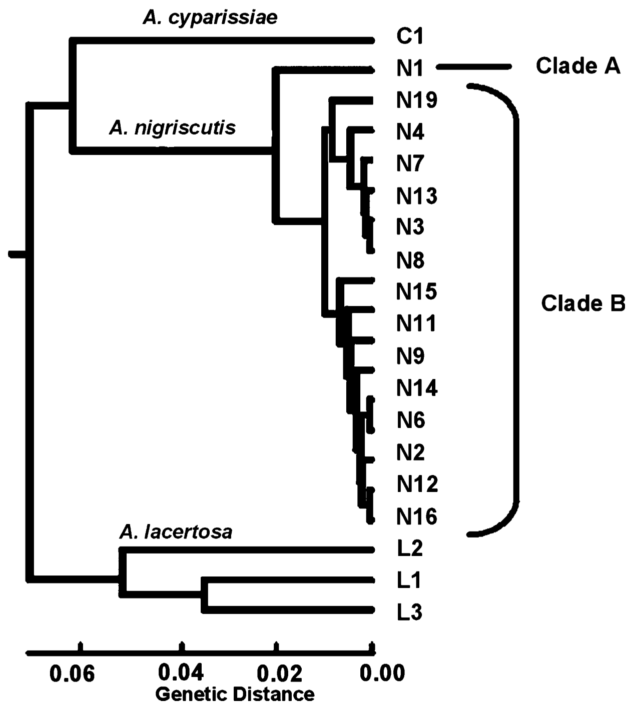


Fig. 1. Tree from RestSite genetic distance matrix of *A. nigriscutis* mtDNA haplotypes. N#-*A. nigriscutis*, haplotype numbers as shown in Table 2. C1-*A. cyparissiae*; L#-*A. lacertosa* haplotypes. Scale is genetic distance.

mtDNA are maternally inherited, an expanding *Wolbachia* infection may be accompanied by the mtDNA genotype(s) that were initially infected. The outcome, known as a *Wolbachia*-associated mitochondrial sweep, is a significant reduction of mtDNA diversity in the infected populations as the mtDNA hitchhikes along with the spreading *Wolbachia* infection (Armbruster et al., 2003; Behura et al., 2001; Rokas et al., 2001; Shoemaker et al., 1999, 2003; Turelli et al., 1992). The mtDNA observations for *A. nigriscutis* are consistent with this scenario. *Wolbachia* may also be associated with less dramatic reductions of variability in the nuclear genome (Telschow et al., 2002).

In general, a strong female bias was found at several of our collection sites, which agrees with previous sex ratio assessments (Brinkman, 1997; Cawthra, 2002; Kazmer, 2001; Nowierski et al., 2001). Kazmer (2001) has also reported that *A. nigriscutis* males do not contain *Wolbachia*. Our observation of two males from Alberta that were haplotype N1 and tested positive for *Wolbachia* appears to counter that claim.The scant number of males we observed makes it impossible for us to comment about the ratio of *Wolbachia* infected to uninfected males. The amount of genetic diversity we found suggests that the spotty establishment of *A. nigriscutis* cannot be attributed to an unusually narrow genetic base of the imported populations. The phenology of the beetles or the effects of an extremely biased sex ratio could potentially have an impact on establishment frequencies. Future collections will attempt to find and target locations that have normal sex ratios. If *Wolbachia* is linked to excess females, then it would be expected that sites with a female-biased sex ratio should have higher proportions of mtDNA haplotype N1. Conversely, a near 50/50 sex ratio should be accompanied by increased numbers of the clade B haplotypes.

It is extremely difficult to evaluate biocontrol possibilities and outcomes when the important variations in the biological control agent cannot be reliably identified. Although the foreign collections of biocontrol agents are tested for their impact on target and non-target organisms, critical genetic studies within and between populations of potential biocontrol agents have not often been performed prior to their release. For example, microsatellite markers for another leafy spurge biocontrol candidate, the leafy spurge gall midge (Spurgia *esulae* Gagne, Cecidomyiidae), have been reported in the past year (Lloyd et al., 2004). The markers represent the beginning of genetic studies with that species. The midge was introduced into North America in 1985, around the same time as the first releases of *Aphthona* were taking place.

Monitoring intraspecific genetic variation of potential classical biological control agents will improve the success of biological control. For example, by comparing genetic profiles of a parasitoid, *Microctonus aethiopoides*, Goldson et al. (2001) were able to identify a foreign source of the biocontrol agent that was better than the population already established in New Zealand. Similar identification of locally adapted strains of *Aphthona* may be possible by linking ecological performance to observed haplotypes. The population genetic issues for introduced natural enemies are essentially the same as those for invasive species, the practical difference being that in the former, the "invasiveness" is to be encouraged and in the latter, "invasiveness" is trying to be throttled (Sakai et al., 2001).

These results also provide a molecular basis for identifying *A. nigriscutis* larvae which will be useful for ecological studies of interspecific competition especially at larval feeding strategies. *Aphthona* larvae are the key damaging stage for leafy spurge plants; however, to date most studies of beetle populations have been of adult beetles because of ease of collection and availability of taxonomic keys (LeSage and Paquin, 1996, 1997). Identification of *Aphthona* larvae to species has not been possible. Our results with *A. nigriscutis* in combination with three other *Aphthona* species (unpublished) will provide a PCR basis for identification of the immature insects.

Little is known about what effect a *Wolbachia* infection may have on the efficacy of a biocontrol release (Mochiah et al., 2002). Kazmer (2001) has suggested that *Aphthona* population growth rates may be slower because of reduced female insemination rates. The fact that both infected and uninfected populations exist at the *A. nigriscutis* release sites indicates that it may be possible to separate uninfected individuals and establish *Wolbachia* free populations at isolated sites to see if they are more effective for leafy spurge control than the current predominantly infected populations. There is also the potential for curing *Wolbachia* infected individuals using antibiotics or heat treatments. This would retain the current genetic base of the established populations. If *Wolbachia* infections are shown to have a negative effect on biological control programs for leafy spurge in North America, new collections from Europe would be another potential source of *Wolbachia*-free insects that would introduce new insect genetic material as well. At this stage some caveats apply. There are other microorganisms that have been shown to affect insect sex ratios (Zchori-Fein and Perlman, 2004). *Wolbachia* is the best candidate here because the insects carry it, but possible affects by other species cannot be ruled out. Additionally, a variety of host plant adaptations could also explain the effective establishment of *Aphthona* beetles at some locations but not others. One intriguing hypothesis suggests that plants can rapidly switch defense strategies between repelling specialized herbivores and repelling generalist herbivores especially when moved to a new environment (Müller-Schärer et al., 2004). The interaction between the three genomes (beetle, microbe, and plant) is likely to be complex.

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## References

Anderson, G.L., DelFosse, E.S., Spencer, N.R., Prosser, C.W., Richard, R.D., 1999. Biological control of leafy spurge: an emerging success story. In: Proceedings, X International Biological Control Symposium, Bozeman, Montana, July 4–9.

Armbruster, P., Damsky, W.E., Giordano, R., Birungi, J., Munstermann, L.E., Conn, J.E., 2003. Infection of new- and old-world *Aedes albopictus* (Diptera: Culicidae) by the intracellular parasite *Wolbachia*: implications for host mitochondrial DNA evolution. J. Med. Entomol. 40, 356–360.

Ballard, J.W.O., Chernoff, B., James, A.C., 2002. Divergence of mitochondrial DNA is not corroborated by nuclear DNA, morphology, or behavior in *Drosophila simulans*. Evolution 56, 527–545.

Behura, S.K., Sahu, S.C., Mohan, M., Nair, S., 2001. *Wolbachia* in the Asian rice gall midge, *Orseolia oryzae* (Wood-Mason): correlation between host mitotypes and infection status. Insect Mol. Biol. 10, 163–171.

Brinkman, M., 1997. Reproductive status of established black dot flea beetle (*Aphthona nigriscutis*) populations in South and North Dakota. Leafy Spurge News 19 (2), 2.

Cawthra, L., 2002. Causes and consequences of female-biased sex ratios in *Aphthona nigriscutis* Foudras, a biological control agent of leafy spurge. M.S. thesis, University of Wyoming.

Cheung, W.Y., Hubert, N., Landry, B., 1993. A simple and rapid DNA microextraction method for plant, animal, and insect suitable for RAPD and other PCR analysis. PCR Methods Appl. 3, 69–70.

Felsenstein, J., 1993. PHYLIP (Phylogeny Inference Package) version 3.6a2. Distributed by the author. Department of Genetics, University of Washington, Seattle.

Flaherty, C., 2001. Finding a home on the range-for beetles. Leafy Spurge News 23 #4, 3–4. Team Leafy Spurge web site (<http://www.team.ars.usda.gov/>).

Goldson, S.L., Phillips, C.B., McNeill, M.R., Proffitt, J.R., Cane, R.P., 2001. Importation to New Zealand quarantine of a candidate biological control agent of clover root weevil. N. Z. Plant Protect. 54, 147–151.

Hansen, R.W., Richard, R.D., Parker, P.E., Wendel, L.E., 1997. Distribution of biological control agents of leafy spurge (*Euphorbia esula* L.) in the United States: 1988–1996. Biol. Control 10, 129–142.

Hopper, K.R., Roush, R.T., Powell, W., 1993. Management of genetics of biological control introductions. Annu. Rev. Entomol. 38, 27–51.

Jeyaprakash, A., Hoy, M.A., 2000. Long PCR improves *Wolbachia* DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. Insect Mol. Biol. 9, 393–405.

Jiggins, F.M., Bentley, J.K., Majerus, M.E.N., Hurst, G.D.D., 2001. How many species are infected with *Wolbachia*? Cryptic sex ratio distorters revealed to be common by intensive sampling. Proc. R. Soc. Biol. Sci. Ser. B 268, 1123–1126.

Jonsen, I.D., Bourchier, R.S., Roland, J., 2001. The influence of matrix habitat on *Aphthona* flea beetle immigration to leafy spurge patches. Oecologia 127, 287–294.

Kazmer, D.J., 2001. Causes and consequences of female-biased sex ratios in *Aphthona* flea beetles. Abstract: Spurgefest II. Team Leafy Spurge, Medora, ND, June 19–21. <http://www.team.ars.usda.gov/symposium/2001/five.html/>.

LeSage, L., Paquin, P., 1996. Identification keys for *Aphthona* flea beetles (Coleoptera: Chrysomelidae) introduced in Canada for the control of spurge (*Euphorbia* spp., Euphorbiceae). Can. Entomol. 128, 593–603.

LeSage, L., Paquin, P., 1997. Erratum: Identification keys for *Aphthona* flea beetles (Coleoptera: Chrysomelidae) introduced in Canada for the control of leafy spurge (*Euphorbia* spp. Eurphorbiaien). Can. Entomol. 129, 201.

Lloyd, C.J., Norton, A.P., Hufbauer, R.A., Bogdanowicz, S.M., Nissen, S.J., 2004. Microsatellite isolation from the gall midge *Spurgia capitigena* (Diptera: Cecidomyiidae), a biological control agent of leafy spurge. Mol. Ecol. Notes 4, 605–607.

Miller, J.C., 1991. RESTSITE, ver. 1.2, Institute of Molecular Evolutionary Genetics, Penn. State Univ.

Mochiah, M.B., Ngi, S.A.J., Overholt, W.A., Stouthamer, R., 2002. *Wolbachia* infection in *Cotesia sesamiae* (Hymenoptera: Braconidae) causes cytoplasmic incompatibility: implications for biological control. Biol. Control 25, 74–80.

Müller-Schärer, H., Schaffner, U., Steinger, T., 2004. Evolution in invasive plants: implications for biological control. Trends Ecol. Evol. 19, 417–422.

Mundal, D.A., Olson, D.L., Carlson, R.B., 1999. The effect of *Aphthona* spp. flea beetle (Coleoptera: Chrysomelidae) larval feeding on leafy spurge, *Euphorbia esula* L., root systems and stem density in North Dakota: 1986–96. Team Leafy Spurge Publications. <http://www.team.ars.usda.gov/teampub/teampublications.html/>, 65pp.

Nowierski, R., Kazmer, D., Horvath, D., Hansen, R. 2001. Ecological barriers for the establishment and population increase of flea beetles on leafy spurge. Abstract: Spurgefest II. Team Leafy Spurge, Medora, ND, June 19–21. <http://www.team.ars.usda.gov/symposium/2001/six.html/>.

Roehrdanz, R.L., 1995. Amplification of complete insect mitochondrial genome in two easy pieces. Insect Mol. Biol. 4, 169–172.

Roehrdanz, R.L., Degrugillier, M.E., 1998. Long sections of mitochondrial DNA amplified from fourteen orders of insects using conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 91, 771–778.

Rokas, A., Atkinson, R.J., Brown, G.S., West, S.A., Stone, G.N., 2001. Understanding patterns of genetic diversity in the oak gallwasp *Biorhiza pallida*: demographic history or a *Wolbachia* selective sweep? Heredity 87, 294–304.

Sakai, A.K., Allendorf, F.W., Holt, J.S., Lodge, D.M., Molofsky, J., With, K.A., Baughman, S., Cabin, R.J., Cohen, J.E., Ellstrand, N.C., McCauley, D.E., O'Neil, P., Parker, I.M., Thompson, J.N., Weller, S.G., 2001. The population biology of invasive species. Annu. Rev. Ecol. Syst. 32, 305–332.

Shoemaker, D., Katju, V., Jaenike, J., 1999. *Wolbachia* and the evolution of reproductive isolation between *Drosophila recens* and *Drosophila subquinaria*. Evolution 53, 1157–1164.

Shoemaker, D.D., Keller, G., Ross, K.G., 2003. Effects of *Wolbachia* on mtDNA variation in two fire ant species. Mol. Ecol. 12, 1757–1771.

Stouthamer, R., Breeuwer, J.A., Hurst, G.D., 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Annu. Rev. Microbiol. 53, 71–102.

Team Leafy Spurge. web site <http://www.team.ars.usda.gov/>.

Telschow, A., Hammerstein, P., Werren, J., 2002. The effect of *Wolbachia* on genetic divergence between populations: models with two-way migration. Am. Nat. 160, S54–S66.

Turelli, M., Hoffmann, A.A., McKechnie, S.W., 1992. Dynamics of cytoplasmic incompatibility and mtDNA variation in natural *Drosophila simulans* populations. Genetics 132, 713–723.

Vala, F., Egas, M., Breeuwer, J.A.J., Sabelis, M.W., 2003. *Wolbachia* affects oviposition and mating behaviour of its spider mite host. J. Evol. Biol. 17, 692–700.

VanBorm, S., Wenseleers, T., Billen, J., Boomsma, J.J., 2001. *Wolbachia* in leafcutter ants: a widespread symbiont that may induce male killing or incompatible matings. J. Evol. Biol. 14, 805–814.

Werren, J.H., 1997. Biology of *Wolbachia*. Annu. Rev. Entomol. 42, 587–609.

Werren, J.H., Windsor, D., Guo, L.R., 1995a. Distribution of *Wolbachia* among neotropical arthropods. Proc. R. Soc. Lond. B 262, 197–204.

Werren, J.H., Zhang, W., Guo, L.R., 1995b. Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proc. R. Soc. Lond. B 261, 55–71.

Zchori-Fein, E., Perlman, S.J., 2004. Distribution of the bacterial symbiont Cardinium in arthropods. Mol. Ecol. 13, 2009–2016.

Zhou, W., Rousset, F., O'Neill, S.L., 1998. Phylogeny and PCR-classification of *Wolbachia* strain using wsp gene sequences. Proc. R. Soc. Lond. Ser. B 265, 509–515.